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Antibody to GBV-C Second Envelope Glycoprotein (Anti-GBV-C E2): Is It a Marker for Immunity?

Howayda M. Hassoba,[1] Norah A. Terrault,[1] Ahmed M. El-Gohary,[1] Christi Scheffel,[2] Cynthia Jou,[2] John Brackett,[2] Jeff Hunt,[2] Sheng Chang Lou,[2] and Teresa L. Wright[1]*

[1]*Department of Medicine, Department of Veterans Affairs Medical Center, University of California, San Francisco, California*
[2]*Abbott Laboratories, Chicago, Illinois*

The clinical significance of GB virus C (GBV-C E2) antibody is under investigation. The prevalence rates of GBV-C RNA and antibody to GBV-C E2 glycoprotein were determined in a population of 123 Egyptian anti-hepatitis C virus (HCV)-positive patients with chronic liver disease (CLD) who had not been treated previously with interferon. Sera were tested for GBV-C RNA by the LCx assay (Abbott Laboratories, North Chicago, IL), and for GBV-C E2 antibody by enzyme immunoassay. GBV-C RNA was present in 11.4% of patients. GBV-C E2 antibody was detected in 55.9% of GBV-C RNA-negative patients and in 2.2% of GBV-C RNA-positive patients (P = 0.006). GBV-C RNA was associated significantly with a history of schistosomiasis (relative risk [RR] = 5.83, 95% confidence interval [CI] 1.99–17.14, $P < 0.005$) but not with parenteral risk factors. The presence of GBV-C E2 antibody was not associated with age, gender, parenteral risk factors, schistosomal infection, or HCV viremia. The HCV genotype and level of viremia were similar in GBV-C anti-E2-positive and negative patients. There was a trend toward more severe histological disease with anti-E2 seropositivity (RR = 1.45, 95% CI 0.89–2.45, P = 0.11), an association which was independent of the evidence of schistosomiasis. It is concluded that GBV-C infection is common among HCV-infected Egyptian patients with CLD due to HCV infection. A significant negative correlation between the GBV-C viremia and GBV-C E2 antibody suggests that an antibody response is associated with viral clearance. This antibody response presumably occurs spontaneously, as none of the patients had received antiviral therapy. The unexpected association between GBV-C RNA and schistosomiasis suggests that nonparenteral or occult parenteral routes of GBV-C infection are likely to be important. ***J. Med. Virol. 53:354–360, 1997.***

KEY WORDS: schistosomiasis; hepatitis C; chronic liver disease; Egyptian population

INTRODUCTION

Using immunoassays and advanced molecular techniques, it is evident that 10–20% of hepatitis cases are caused by non-A to E agents. Two new flavi-like viruses (GBV-A and GBV-B) derived from tamarins inoculated with GB agents were described in 1995, and a third genus was isolated subsequently and given the name GBV-C [Simons et al., 1995]. GBV-C was isolated from human sera using consensus primers derived from GBV-A and GBV-B [Kuo et al., 1989; Dawson et al., 1992]. The genomes of these three new GB viruses are single-stranded, positive-sense RNA of approximately 9.5 kb. Almost simultaneous with the discovery of GBV-C, a novel RNA virus, designated hepatitis G virus (HGV), was cloned and sequenced by Linnen et al. [1996]. GBV-C and HGV have approximately 86% identity at the nucleotide level and 95–100% identity at the amino acid level, providing strong evidence that the two viruses are probably variants of the same virus [Schlauder et al., 1995; Alter, 1996; Shao et al., 1996]. On the other hand, the presence of only 26–30% homology at the amino acid level with the hepatitis C virus (HCV), another member of the *Flaviviridae* family, suggests that GBV-C and HGV are distinct viruses from HCV [Simons et al., 1995; Thomas, 1995; Zuckerman, 1996].

In patients with chronic hepatitis C infection, the antibody against HCV core protein (c22) is the earliest and the most consistently reactive antigen in the HCV enzyme immunoassay (EIA). However, this is not true for GBV-C. The core nucleoprotein of GBV-C/HGV

*Correspondence to: Gastroenterology Section (111B), Veterans Administration Medical Center, 4150 Clement St., San Francisco, CA 94121. Fax: (415) 750-2196.

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seems to be either absent or defective [Erker et al., 1996; Leary et al., 1996a; Linnen et al., 1996]. The manner in which this defective or absent GBV-C core protein influences the pathogenesis of the disease is unknown. However, this defect may explain the difficulties encountered in the development of specific and sensitive antibody assays for GBV-C and HGV. For the most part, diagnosis is dependent currently upon polymerase chain reaction (PCR) assays using primers from conserved regions of the genome.

Antibody assays based upon the envelope region of HCV (E2) are correlated positively with the presence of HCV RNA (97.3%) in serum, suggesting that anti-E2 may be a useful marker of HCV infection [Lesniewski et al., 1995]. Recently, an antibody assay based upon the envelope region of GBV-C has been developed, and the relationship between GBV-C E2 antibody and GBV-C RNA is under active investigation, though the clinical significance of GBV-C E2 antibody has yet to be determined [Pilot-Matias et al., 1996; Dille et al., 1997].

Parenteral exposure has been suggested to be the major mode of GBV-C transmission, an observation which is supported by the frequent coexistence of GBV-C with other parenterally acquired viruses such as HCV and hepatitis B virus (HBV) [Simons et al., 1995; Leary et al., 1996; Linnen et al., 1996; Marrone et al., 1996]. In Egypt, the first reported prevalence of hepatitis C infection among patients with non-A, non-B (NANB) chronic liver disease was 66.8% [El-Zayadi et al., 1992], and early reports related 35–70% of acute hepatitis cases in Egypt to NANB viruses [Bassily et al., 1987]. Additionally, markers of HCV alone or HCV and HBV coinfection are present in 30% and 40%, respectively, of patients diagnosed with hepatocellular carcinoma [Darwish et al., 1993]. Thus, HCV infection is a major health problem among the Egyptian population. The prevalence of GBV-C has not been determined previously in an Egyptian population. However, from the high prevalence of HCV and HBV in this region, one would predict that the prevalence of GBV-C infection also would be high. In the present study, we sought to determine the prevalence of GBV-C virus (by PCR) and GBV-C E2 antibody in a population of patients with chronic HCV infection and to examine the clinical and epidemiological variables associated with GBV-C RNA and anti-E2 positivity.

PATIENTS AND METHODS

Study Population

One hundred twenty-three consecutive Egyptian patients with chronic HCV-associated liver disease attending the Liver Clinic in Ismailia University Hospital in Egypt during a period extending from November 1994 to December 1995 were selected for inclusion in this study. Inclusion criteria were *(i)* persistent elevation of liver transaminases for at least 6 months, *(ii)* HCV antibody-positive by second-generation EIA, *(iii)* negative serology for hepatitis B surface antigen (HBsAg), and *(iv)* no previous antiviral treatment for HCV. Patients were interviewed for the following demographic information: age, gender, risk factors for viral acquisition, and date of first exposure to HCV risk factors. The risk factors for viral acquisition evaluated were a previous history of *(i)* blood transfusion, *(ii)* surgical procedures, *(iii)* dental procedures, *(iv)* schistosomal infection (previous or active), *(v)* injections (intramuscular or intravenous), and *(vi)* dialysis. Liver tests (alanine aminotransferase [ALT], aspartate aminotransferase [AST], serum albumin, and total bilirubin) were obtained on all patients.

Laboratory Methods

GBV-C

GBV-C RNA detection (PCR). For GBV-C RNA extraction, the nucleic acid target was extracted from 25 μl serum using 280 μl buffer AVL/carrier RNA (QIAamp'HCV kit), for at least 10 min at room temperature. The GBV-C RNA was precipitated in an equal volume of 100% ethanol and the solution transferred into a QIAamp spin column and centrifuged at 6,000 *g* for 1 min. The column was transferred to a clean 2 ml collection tube and washed twice with 500 μl AW buffer (QIAamp'HCV kit) by spinning at 6,000 *g* for 1 min. Molecular biology grade water (100 μl, 5 prime → 3 prime, INC™) preheated to 80°C was added to the filter, followed by a final spin at 6,000 *g* for 1 min to elute the RNA. Purified RNA was kept on ice and amplified within 4 hr of purification. Six controls were included in each run: 2 negative controls, 2 low positive controls, and 2 high positive controls.

The Abbott (North Chicago, IL) LCx probe system uses PCR for nucleic acid amplification. Reverse transcription was undertaken at 60°C for 30 min. The PCR denaturation step was carried out at 94°C for 40 sec, followed by annealing at 63°C for 1 min for a total of 35 cycles. The reaction mix then was heated at 97°C for 5 min to denature the amplification products. The GBV-C-specific probe was hybridized to the single-stranded amplicon while the reaction mix was cooled rapidly to 12°C.

The probe/PCR complex was detected using a microparticle enzyme immunoassay (MEIA), in which submicron-sized microparticles coated with antibodies "captured" the complex, forming immune complexes. Two different haptens attached to the probe. One of the haptens (capture hapten) allowed the probe-hybridized amplicon to be captured on a solid phase (glass fiber matrix). After washing, only the immune complexes were retained, while the unbound materials were removed. The captured complexes were detected using an antibody–enzyme conjugate (alkaline phosphatase-labeled antibody) directed against the second hapten (detection hapten). A second wash removed unbound conjugate. Addition of the flourogenic substrate (4-methylumbelliferyl phosphate, MUP) and its subsequent hydrolysis by the bound conjugate (as a catalyst) into MU produced a fluorescent emission. The MEIA optics measured the rate at which MU was generated.

TABLE I. GBV-C RNA and E2 Antibody in the Study Population

	E2 Ab$^+$	E2 Ab$^-$	Total
GBV-C RNA$^+$	2 (2.2%)	9 (9.6%)	11 (11.8%)
GBV-C RNA$^-$	52 (55.9%)	30 (32.3%)	82 (88.2%)
Total	54 (58.1%)	39 (41.9%)	93 (100%)

r = 0.31, P = 0.006 (Fisher's exact test).

GBV-C E2 antibody detection. The immunoassay for detection of human antibody elicited against GBV-C was carried out at Abbott Laboratories. Briefly, a glycosylated form of GBV-C E2 protein was purified and used as an antigenic target for detection of human anti-GBV-C antibody. An indirect immunoassay was developed which employed E2 on a solid phase to capture antibody from human serum or plasma, followed by the addition of an enzyme-conjugated antihuman antibody for color development. Positive or gray zone results were confirmed for each serum by a sandwich type immunoassay using the E2 protein [Lou et al., in press].

HCV detection

HCV antibody. HCV antibodies were detected by second-generation enzyme-linked immunoassay (EIA-2) according to the manufacturer's instructions (Ortho Diagnostic Systems, Raritan, NJ). Results were expressed as the absorbance value divided by the cutoff value, and specimens were considered to be reactive if the determination was greater than or equal to the cutoff value. Some of the EIA-2-reactive samples were tested by second-generation recombinant immunoblot assay (RIBA-2™) (Chiron Corporation, Emeryville, CA).

HCV-RNA detection. HCV-RNA was extracted from 50 μl of serum using the proteinase K/phenol-chloroform method; cDNA was generated from HCV RNA using methods previously described [Wright et al., 1992].

HCV RNA quantitation. This was carried out using the bDNA assay (Quantiplex HCV 2.0™, Chiron) as described previously [Detmer et al., 1996]. The lower limit of detection of this assay was 0.2 MEq/ml.

HCV genotyping. This was undertaken by restriction fragment length polymorphism (RFLP) of the 5′ untranslated region (UTR) region of the virus as described previously [Zhou et al., 1996].

Histological Methods

Liver biopsies were available in 76 (62%) of the study subjects. Each biopsy was assessed for *(i)* histological evidence of schistosomiasis and *(ii)* severity of disease (inflammation and fibrosis). Evidence of schistosomal liver disease included bilharzial hepatitis fibrosis (BHF), BHF with chronic active hepatitis, and BHF with cirrhosis. Disease severity was classified as mild, moderate, or severe based upon the inflammatory component. Patients with a diagnosis of chronic persistent hepatitis, lobular hepatitis, or mild chronic active hepatitis were classified as "mild." Patients with moderate chronic active hepatitis were classified as "moderate." Patients with severe chronic active hepatitis or cirrhosis (active or inactive) were classified as "severe."

Statistical Analysis

Values are expressed as median, range, and percentages as appropriate. Continuous variables were compared using the Mann-Whitney U test (nonparametric test) and categorical variables compared using the χ^2 or Fisher's exact test. The predictor variables examined were age, gender, risk factors (known/unknown), ALT, HCV RNA (present/absent), HCV genotype (type 4 vs. others), HCV bDNA levels, schistosomiasis (present/absent), and histological disease severity (mild, moderate vs severe). Individual risk factors evaluated included blood transfusion, history of injections, dental procedures, surgery, and evidence of schistosomiasis. The outcome variables were GBV-C RNA (present/absent) and GBV-C E2 antibody (present/absent). Unconditional logistic regression was used to evaluate the independent effects of GBV E2 antibody and schistosomiasis on disease severity. The correlation between GBV-C RNA and GBV-C E2 antibody was tested using Spearman's rank correlation (r).

RESULTS

Study Population Characteristics

One hundred twenty-three patients met the inclusion criteria. The ratio of males to females was 2:1, and the median age at the time of evaluation was 39 years (range 12–69). Known risk factors for HCV acquisition were elicited in 85 (69.1%) patients: 39 (31.7%) had a history of blood transfusion (including dialysis), 20 (16.3%) a history of major surgery, 11 (8.9%) a history of dental procedures, 3 (2.4%) a history of previous injections, and 12 (9.8%) a history or histological evidence of schistosomiasis. In 38 (30.9%) patients, no known risk factors for HCV acquisition could be elicited. Genotype 4 was the most common type and was present in 78.4% of patients; types 1a and 1b were present in 2% and 11.4%, respectively.

Relationship Between GBV-C RNA and E2 Antibody

The prevalence of GBV-C RNA was 14/123 (11.4%). Among the 123 patients who were tested for GBV-C RNA, serum from 93 patients was available for anti-E2 antibody testing. The E2 antibody was reactive in 54 (58% patients). Of the 14 patients with GBV-C RNA detectable in serum, 11 had serum samples available for GBV-C E2 antibody testing. Anti-GBV-C E2 was nonreactive in nine patients and reactive in two patients. In the 82 patients without GBV-C RNA detectable in the serum, 52 (63.4%) were reactive for the E2 antibody, while 30 (36.6%) were nonreactive. The pres-

TABLE II. Clinical and Virological Features of Patients With and Without GBV-C RNA

Clinical features	GBV-C RNA-positive (n = 14)	GBV-C RNA-negative (n = 109)	*P*
Age (years) median (range)	39 (19–50)	38 (12–69)	0.31[d]
Gender (M:F)	9:5	73:36	0.84[e]
Known risk factors			
Schistosomiasis	5 (35.7%)	7 (6.4%)	**0.005[e]**
Blood transfusion	3 (21.4%)	36 (33%)	0.23[e]
Surgery	1 (7.1%)	19 (17.4%)	0.26[e]
Dental procedures	1 (7.1%)	10 (9.1%)	0.62[e]
Injections	0	3 (2.7%)	0.68[e]
ALT XULN IU/l median (range)	1.2 (0.3–2.6)	2.0 (0.1–6.8)	0.24[d]
HCV RNA (PCR)			0.36[a,e]
Positive	9 (64.2%)	66 (60.5%)	
Negative	3 (21.4%)	41 (37.6%)	
Indeterminate	2 (14.2%)	2 (1.8%)	
HCV genotype (n = 79)			0.56[b,e]
Type 4	9 (81.8%)	53 (77.9%)	
Type 1a or 1b	1 (9%)	14 (20.5%)	
Nontypable	1 (9%)	1 (1.4%)	
HCV bDNA (MEq/ml) median (range) (n = 31)	2.05 (0.2–8.0%)	2.61 (0.2–29.4)	0.62[d]
Schistosomiasis on biopsy (n = 105)			0.21[e]
Present	8 (57.1%)	36 (39.5%)	
Absent	6 (42.8%)	55 (60.4%)	
Disease severity (n = 105)			0.30[c,e]
Mild	5 (35.7%)	21 (23%)	
Moderate	4 (28.5%)	24 (26.3%)	
Severe	5 (35.7%)	46 (50.5%)	

[a]HCV positive versus negative (indeterminate excluded).
[b]Type 4 vs. non-type 4 (nontypable excluded).
[c]Mild and moderate (combined) vs. severe.
[d]Mann-Whitney U-test.
[e]χ^2 or Fisher's exact test.

ence of virus was correlated negatively with the presence of antibody (r = −0.31, $P = 0.006$; Table I).

Clinical and Virological Features in GBV-C RNA-Positive Patients

There were no significant differences between the age, gender, mean ALT level, HCV RNA detectability, level of HCV viremia, HCV genotype, or disease severity in GBV-C RNA-positive and GBV-C-negative patients (Table II). The distribution of risk factors was significantly different between the two populations, with GBV-C viremic patients reporting a greater frequency of schistosomal infection (relative risk [RR] = 5.83, 95% confidence interval [CI] 1.99–17.14, $P = 0.005$, χ^2 test) than GBV-C-negative patients.

Clinical and Virological Features of GBV-C E2 Antibody-Positive Patients

There were no significant differences between the age, gender, mean ALT level, HCV RNA detectability, HCV level of viremia, genotype, or risk factors for acquisition in GBV-C E2 antibody-positive patients compared to GBV-C E2 antibody-negative patients (Table III). A trend toward more severe histological disease among GBV-C E2 antibody-positive patients was evident (RR = 1.45, 95% CI 0.89–2.36, $P = 0.11$, χ^2 test). This relationship was independent of the presence or absence of schistosomiasis on biopsy.

DISCUSSION

To our knowledge, this is the first report to study *(i)* the prevalence of GBV-C RNA and GBV-C E2 antibody in an Egyptian population and *(ii)* the contribution of schistosomiasis, HCV infection, and other risk factors to the prevalence of GBV-C E2 antibody. The prevalence of GBV-C RNA in this population of Egyptian patients with chronic liver disease due to HCV infection was 11.4%. This point prevalence is within the range of the reported prevalences for GBV-C in HCV-infected populations from other countries (6.6–16%) [Oshita et al., 1996; Pawlotsky et al., 1996; Tanaka et al., 1996]. The variability in prevalence likely reflects differences in the characteristics of the patient populations, such as age, stage of disease, and previous interferon treatment. For example, we have shown previously that the prevalence of HGV among HCV-infected patients referred for liver transplantation was 24%, suggesting that the prevalence of GBV-C/HGV infection among patients with advanced disease may be higher than among those with early stages of liver disease [Berenguer et al., 1996].

A lack of information regarding the natural history of acute and chronic infection and the duration of GBV-C infection further complicates the interpretation of the prevalence data. Examination of the relationship between GBV-C RNA and the antibody to E2 sheds some light on this issue. A high prevalence of E2 antibody was found among those negative for GBV-C RNA,

TABLE III. Clinical and Virological Features of Patients With and Without GBV-C anti-E2

Clinical features	Anti-GBV-C E2-positive (n = 54)	Anti-GBV-C E2-negative (n = 39)	*P*
Age (years) median (range)	37.5 (12–69)	38 (18–49)	0.31[d]
Gender (M:F)	39:15	27:12	0.80[e]
Known risk factors			
Blood transfusion	17 (31.4%)	11 (28.2%)	0.98[e]
Surgery	10 (18.5%)	5 (12.8%)	0.36[e]
Schistosomiasis	5 (9.2%)	4 (10.2%)	0.45[e]
Dental	4 (7.4%)	5 (12.8%)	0.27[e]
Injections	2 (3.7%)	1 (2.5%)	0.64[e]
ALT XULN (IU/l) median (range)	1.8 (0.1–6.8)	1.6 (0.3–6.8)	0.24[d]
HCV PCR (n = 93)			0.80[a,e]
Positive	28 (51.8%)	18 (46.1%)	
Negative	26 (48.1%)	17 (43.5%)	
Indeterminate	0	4 (10.2%)	
HCV genotype (n = 67)			0.16[b,e]
Type 4	23 (79.3%)	17 (80%)	
Type 1a or 1b	6 (20.7%)	2 (9.5%)	
Nontypable	0	2 (9.5%)	
HCV bDNA MEq/ml) median (range) (n = 31)	1.94 (0.21–11.5)	2.15 (0.28–29.4)	0.62[d]
Schistosomiasis on biopsy (n = 76)			0.51[e]
Present	17 (38.6%)	14 (43.7%)	
Absent	27 (61.3%)	18 (56.2%)	
Histologic severity (n = 76)			0.11[c,e]
Mild	10 (22.7%)	7 (21.8%)	
Moderate	8 (18.1%)	12 (37.5%)	
Severe	26 (59%)	13 (40.6%)	

[a]HCV-positive vs. -negative (indeterminate excluded).
[b]Type 4 vs. non-type 4 (nontypable excluded).
[c]Mild and moderate (combined) vs. severe.
[d]Mann-Whitney U-test.
[e]χ^2 or Fisher's exact test.

and conversely, anti-E2 antibody was uncommon in patients with detectable viremia. This suggests that the development of antibody is associated with viral clearance. Since none of the patients in our series had ever received antiviral therapy, clearance of GBV-C infection would seem to have occurred spontaneously. If true, the "natural history" of GBV-C infection would appear to be quite different from that of HCV since spontaneous clearance of the latter infection is uncommon [Alter, 1995]. Whether the presence of HCV infection in some way enhances the clearance of GBV-C infection cannot be discerned from our cross-sectional study. Longitudinal study of coinfected patients would be required to address this issue.

Although the majority of patients with GBV-C E2 antibody were without GBV-C RNA, suggesting that the antibody represents resolution of viremia, in two (2.2%) patients GBV-C E2 antibody and GBV-C RNA were detectable simultaneously. There are several interpretations of this finding. This may represent the phase of viral clearance since antibody and virus are detected concurrently in many viral infections as they resolve. Confirmation of this hypothesis would require follow-up serum samples; one would expect loss of GBV-C RNA with continued follow-up and persistence of GBV-C E2 antibody [Pilot- Matias et al., 1996]. Alternatively, since it is not known whether the presence of E2 antibody protects against subsequent GBV-C infection, the presence of GBV-C RNA and GBV-C E2 antibody within the same individual may represent reinfection with another strain of GBV-C after previous exposure. A recent report of HGV-E2 antibody prevalence among injection drug users does not support this latter hypothesis [Tacke et al., 1997]. These investigators found that prevalence of HGV RNA was higher in users of 5 years or less and prevalence of HGV-E2 antibody was higher in users of greater than 5 years, suggesting that individuals who develop a humoral response may be protected against subsequent infection.

The biological role of the GBV-C E2 protein is not understood. In general, envelope proteins are believed to be important for eliciting an effective neutralizing antibody response [Schlesinger et al., 1985]. However, this is not the case for at least one member of the *Flaviviridae* family, HCV. Although all patients with HCV infection have evidence of humoral immunity, the antibody response appears to be inadequate for neutralizing virus and conferring long-lasting protection against subsequent infection. Cross-challenge studies in the chimpanzee demonstrated that prior infection with HCV fails to provide protection against inoculation with either heterologous or even homologous strains of virus [Farci et al., 1992]. The failure of antibody to protect against subsequent infection has been attributed to rapid mutations in HCV with resultant "immune escape." Successful induction of humoral immunity appears to be isolate-specific and transient. The results of our study suggest that the presence of GBV-C E2 antibody is indicative of immunity as the majority of patients with antibody did not have circu-

lating virus. This suggests that the humoral response to GBV-C is quite different from that of HCV.

There was no correlation between GBV-C and the detection and level of HCV viremia or HCV genotype. A possible viral interaction is suggested by the trend toward more severe disease in HCV-infected patients with GBV-C E2 antibody. This effect was independent of the histological effects of schistosomiasis. A previous study comparing patients with HCV to patients with HCV/HGV coinfection found no difference in histological severity of disease [Bralet et al., 1997]. If an interaction is present, the precise mechanism of the interaction cannot be discerned from our data. There were no differences between the levels of HCV viremia in patients with and without GBV E2 antibody.

A significant correlation was found between the presence of GBV-C RNA and evidence of previous schistosomal infection. This was the only significant risk factor associated with the presence of GBV-C viremia. A strong association between schistosomal infection and HCV infection was highlighted previously, and the proposed mechanism of HCV acquisition in these instances has been related to treatment of schistosomiasis by injection therapy. In our study, few patients with a history of schistosomiasis gave a history of injections, so the relationship between GBV-C viremia and schistosomiasis may be related to nonparenteral risk factors or to unrecognized parenteral routes of acquisition. The high prevalence of GBV-C/HGV (1.7%) among blood donors compared to both HCV and HBV suggests that other routes of transmission may be important for acquisition of GBV-C/HGV [Linnen et al., 1996].

In summary, a high prevalence of GBV-C infection in an Egyptian population has been demonstrated. Moreover, an inverse relationship was evident between the presence of antibody (anti-GBV-C E2) and the presence of GBV-C viremia, suggesting an effective humoral response. The duration of the antibody response and the protective effect of antibody against subsequent exposure to GBV-C infection will need to be delineated in future studies.

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